Exhibit 3

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Title: Esters of 5-aminolevulinic acid as photosensitizing agents in photochemotherapy

Assistant Commissioner for Patents
Washington DC 20231

Declaration of Kristian Berg under 37 C.F.R. §1.131

I, Kristian Berg of Oslo, Norway, hereby declare:

1. I have held the position of Scientist at the Department of Biophysics, The Norwegian Radium Hospital, Institute for Cancer Research, Oslo, Norway, since 1985 .

2. I have considerable experience of photodynamic therapy having worked in this area for many years. In particular, I have been conducting experimental work with 5-aminolevulinic acid (ALA) and esters of ALA in photodynamic therapy since 1991 and have investigated the differences in effect of ALA and ALA esters when used in PDT and also the mechanisms of action of ALA and ALA esters in PDT.

3. Photochemotherapy, or photodynamic therapy (PDT) is a relatively recent technique developed for the treatment of various abnormalities or disorders of the skin or other epithelial organs or mucosa, especially cancers or pre-cancerous lesions, as

well as certain non-malignant lesions for example skin complaints such as psoriasis.
Photochemotherapy involves the application of photosensitizing (photochemotherapeutic) agents to the affected area of the body, followed by exposure to photoactivating light in order to activate the photosensitizing agents and convert them into cytotoxic form, whereby the affected cells are killed or their proliferative potential diminished.

4. A range of photosensitizing agents are known, of which one of the most important are the porphyrins. However, porphyrin based drugs have a number of disadvantages including the fact that they do not readily penetrate the skin when applied topically. To overcome these problems, a natural precursor of PpIX (5-aminolevulinic acid (ALA), formed from succinyl CoA and glycine in the first step of heme synthesis) has been investigated as a photochemotherapeutic agent for certain skin cancers. ALA is able to penetrate the skin to a limited extent and lead to a localised build-up of PpIX. Thus, by applying ALA topically to skin tumours, and then after several hours exposing the tumours to light, a beneficial photochemotherapeutic effect may be obtained.

5. However, whilst the use of ALA represents a significant advance in the art, photochemotherapy with ALA is not always entirely satisfactory. ALA is not able to penetrate all tumours and other tissues with sufficient efficacy to enable treatment of a wide range of tumours or other conditions.

6. We investigated ester derivatives of 5-ALA as potential alternative photochemotherapeutic agents.

Surprisingly, ester derivatives of 5-ALA showed better penetration of cell membranes and also other unexpected advantages over 5-ALA.

7. I am informed that the Examiner examining the US patent application directed to ALA esters for use in PDT is of the opinion that the use of ALA esters for PDT is obvious for a number of reasons. Firstly, it has been suggested that the fact that 5-ALA esters penetrate the skin more easily is obvious since 5-ALA is quite a hydrophilic molecule, whereas the ALA esters are, by their very nature, more hydrophobic. Secondly, it has been suggested that once 5-ALA esters have penetrated the cell, it is obvious that they will, in effect, act in exactly the same way as ALA, as they would be hydrolysed to ALA in a first step i.e. the ALA-ester acts merely as a prodrug.

8. With regard to the first point, i.e. the greater lipophilicity of 5-ALA ester and the suggestion that this would automatically lead to greater penetration of cell membranes than free 5-ALA, this relies on the assumption that ALA and ALA esters would penetrate the membrane of cells by passive diffusion. This assumption is not however valid in view of what is known about the passage of ALA through cell membranes.

9. 5-ALA is an endogenous delta amino acid with structural similarities to the β-amino acids β-alanine and taurine as well as GABA (please see Fig. 5, in Annex 1 attached to this Declaration). Moreover, it is known that particular active transport systems exist for amino acids. This fact is discussed in Annex 1 and in the references (e.g. 17 & 18) cited therein. Thus, it would be expected

that an endogenous amino acid such as 5-ALA would be transported across the plasma membrane via one of the amino acid transport systems. Experiments confirming this are shown in Annex 1 (and in the document comprising Annex 2), where evidence is presented that 5-ALA is transported either by the β-amino or the GABA transport systems and that such transport was energy dependent (as would be expected for an active specific transport system), pH dependent, temperature dependent and Na^+ and Cl^- dependent.

10. However, it would not be expected that non-endogenous derivatives of endogenous amino acids (such as 5-ALA esters) would be transported via such endogenous natural transport systems. This is particularly the case given the fact that evidence supporting this exists in other systems. For example, DOPA (an endogenous amino acid) has an endogenous transport system which effects passage across the blood brain barrier. In contrast, dopamine which is a non-naturally occurring decarboxylation product of the naturally occurring amino acid DOPA, does not pass through the blood brain barrier suggesting that despite small structural changes it is unable to use the DOPA transport system. Consequently, DOPA is used medically, e.g. in the treatment of Parkinson's disease. Furthermore, it has been shown that L-DOPA-esters are not transported in the same way as DOPA and hence also do not cross the blood brain barrier. Thus, a relatively minor change such as esterification, regardless of the increase in lipophilicity of the compound, results in significant alterations in its properties in terms of its transport within the cell. It would therefore be counter-intuitive to make esters of 5-

ALA, because one would expect that this would result in a disturbance in the endogenous transport system, or in any event one would expect that the esters would not be transported across the membrane by the same mechanism. Proof that this expectation is in fact correct is demonstrated in Annex 1. I would also expect that passive diffusion of the ester across the cell membrane, if it occurred, would be less efficient than a natural endogenous active transport mechanism and would not provide sufficient quantities of a photochemotherapeutic agent within the cell for the purposes of PDT. The fact that the uptake of the ALA-ester into cells was demonstrated to be better than the uptake of free 5-ALA was therefore, extremely surprising.

11. With regard to the second point, i.e. that once inside the cell, the ester would act as a prodrug of ALA, I would not dispute the fact that this would be the expectation. However, this expectation was not fulfilled. Firstly it was determined in the course of my studies that ALA-esters do not behave as ALA prodrugs since the mechanism by which ALA-esters induce PpIX does not necessarily involve the complete hydrolysis of ALA-ester to ALA as a first step, and it appears that ALA-methyl ester may itself enter the first step in the endogenous biosynthetic pathway to PpIX. These results, which are presented in Annex 3, were indeed surprising. In view of these results showing that PpIX can be derived from ALA esters without a hydrolysing step to ALA, it is my opinion that the ALA-esters should not be regarded purely as prodrugs. Thus the Examiner's opinion that the use of ALA esters would be obvious is incorrect on two counts. Firstly one would not use esters since to do so would be counter-intuitive and secondly

once within the cell ALA esters do not behave as ALA prodrugs.

12. Not only was it not obvious to use ALA esters in PDT, but unexpected and very surprising properties, demonstrated by my colleagues at The Norwegian Radium Hospital, Institute for Cancer Research, were found to flow from the use of ALA esters relative to ALA in PDT.

13. The first of the unexpected properties of the ALA-esters over free-ALA is the absence of transport in the blood to other tissues, i.e. the lack of systemic distribution displayed by the ALA-ester. Experiments demonstrating this unexpected property were carried out by Professor J. Moan at the Department of Biophysics, The Norwegian Radium Hospital, Institute for Cancer Research. Data showing this unexpected phenomenon are presented in Annex 4, where it is shown that topical administration of ALA on a nude mouse resulted in PpIX formation all over the mouse (i.e. a completely red mouse) whereas topical administration of ALA-ester did not result in any PpIX formation outside the application site. Such properties of the ALA-ester are clearly advantageous for PDT and would not be predicted if the assumption that ALA-ester would simply act as a pro-drug for ALA was correct. Thus, it is my opinion that such properties were surprising.

14. A second unexpected property identified during the course of the research of my colleagues at The Norwegian Radium Hospital, Institute for Cancer Research, into the ALA esters was that the ALA-esters displayed a significantly increased selectivity for tumour tissue versus surrounding

non-tumour tissue. The demonstration of this improved selectivity is shown in Annexes 5, 6 and 7 (the data in Annex 5 having been produced by Professor J. Moan at the Department of Biophysics, The Norwegian Radium Hospital, Institute for Cancer Research, and the data in Annex 7 having been produced by Dr Q. Peng at the Department of Biophysics (until 1999) and the Department of Pathology (from 1999), The Norwegian Radium Hospital, Institute for Cancer Research). Although the data presented in Annexes 5 and 7 is derived from experiments carried out by my colleagues at The Norwegian Radium Hospital, an independent study by Fritsch and coworkers in Germany supported these results by showing preferential enrichment of porphyrins, within the skin of the cancerous lesion as opposed to the normal skin (see Annex 6). Again, such increased selectivity would not be predicted if the ALA-esters were simply acting as prodrugs and as such this property is surprising. Moreover, this surprising property of the ALA-esters is clearly advantageous for PDT.

15. A third unexpected advantageous property which was observed during clinical trials using ALA and ALA esters is that much less severe pain is felt by patients treated with ALA esters than with ALA alone (with ALA alone a slight burning sensation is felt by some patients on irradiation, which is not felt by patients treated with ALA esters). This unexpected phenomenon may be explained by the fact that ALA but not ALA-ester can be transported by the GABA receptor. GABA transporters are located in peripheral neurons, meaning that 5-ALA but not 5-ALA esters should be expected to be preferentially taken up into peripheral nerve endings. This may thus explain the more severe

pain reactions observed during light exposure of basal cell carcinomas lesions after topical application of 5-ALA as compared to 5-ALA methyl ester. This phenomenon is again discussed in Annex 1.

16. I have been provided with a copy of the specification of International patent application No. PCT/GB96/00553 (Publication No. WO96/28412) from which the US patent under examination derives. The other surprising advantages which have been identified for ALA esters relative to ALA are as described on pages 8 to 9. Thus, in addition to the effects described above, ALA esters are better able to penetrate skin and other tissues as compared with ALA and the penetration is deeper and faster. Furthermore the esters have been found to be better enhancers of PpIX production than ALA and PpIX production levels following administration of the ALA esters are higher than with ALA alone.

17. Taken together these results are evidence that ALA esters outperformed ALA in PDT by exhibiting many advantageous effects that could not have been expected.

18. All statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements and the like so made are punishable by fine or imprisonment, or both, under

Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Respectfully submitted

Date 25/5-99

KRISTIAN BERG

ANNEX 1

Manuscript of Rud & Berg entitled "5-aminolaevulinic acid, but not 5-aminolaevulinic acid esters, are transported into cells by γ-aminobutyric acid and β-amino acid carriers"

Doc 8.

5-AMINOLAEVULINIC ACID, BUT NOT 5-AMINOLAEVULINIC ACID ESTERS, ARE TRANSPORTED INTO CELLS BY γ-AMINOBUTYRIC ACID AND β-AMINO ACIDS CARRIERS

by

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Short title: Cellular uptake of 5-aminolaevulinic acid

Abbreviations: 5-ALA, 5-aminolaevulinic acid; PpIX, protoporphyrin IX; PDT, photodynamic therapy; FCS, foetal calf serum; PBS, Dulbecco's phosphate-buffered saline; GABA, γ-aminobutyric acid.

SYNOPSIS

The transport of 5-aminoleavulinic acid (5-ALA) has been studied in a human adenocarcinoma cells line (WiDr) by means of [^{14}C]-labelled 5-ALA. The rate of uptake was saturable following Michaelis-Menten kinetics (K_m = 8-10 mM and V_{max} = 18-20 nmol · (mg protein x h)$^{-1}$) and Arrhenius plot of the temperature-dependent uptake of 5-ALA was characterised by a single discontinuity at 32°C. The activation energy was 112 kJ · mol^{-1} in the temperature range 15-32°C and 26 kJ · mol^{-1} above 32°C. Transport of 5-ALA was Na^+ and partly Cl^- dependent in that the uptake was reduced by 90% and 65% when Na^+ was replaced with choline chloride and Cl^- replaced by NO_3^-/SO_3^- or gluconate, respectively. Stoichiometric analysis revealed a Na^+:5-ALA coupling ratio of 3:1. The substrate specificity of the 5-ALA transporter(s) was analysed by treating the cells with 23 μM [^{14}C]-5-ALA and 10 mM of 20 different α-amino acids. With the exception of valine, methionine and threonine, zwitterionic and basic amino acids inhibited the transport of 5-ALA. 5-ALA methyl ester was not an inhibitor of 5-ALA uptake. The transport was most efficiently inhibited, i.e. by 70-75%, by the β-amino acids β-alanine and taurine and by γ-aminobutyric acid (GABA). Protoporphyrin IX accumulation in the presence of 5-ALA (0.3 mM) was attenuated by 85% in the presence of 10 mM β-alanine, whilst PpIX formation in cells treated with 5-ALA methyl ester (0.3 mM) or 5-ALA hexyl ester (4 μM) was not significantly influenced by β-alanine. Thus, 5-ALA , but not 5-ALA esters, are transported by β-amino acid and GABA carriers in this cell line.

INTRODUCTION

Topical or systemic administration of 5-aminolaevulinic acid (5-ALA) as used in photodynamic therapy, results in accumulation of porphyrins and in particular protoporphyrin IX [1,2]. The initial step in the heme synthesis pathway is the 5-ALA synthase-induced formation of 5-ALA from succinyl-CoA and glycine, and this step is regulated by feedback inhibition by heme [3]. By treating cells with 5-ALA, this negative feedback can be overruled.

The initial step in the 5-ALA-induced synthesis of porphyrins is the penetration of 5-ALA through the plasma membrane. This step may under certain conditions be a rate-limiting factor in the formation of PpIX. This is also in accordance with the observation that esterification of 5-ALA with aliphatic alcohols (C_6 or longer) was found to reduce 30-150 fold the amount of drug needed to reach the same level of PpIX accumulation [4]. However, the mechanisms associated with the uptake of 5-ALA and 5-ALA esters in neoplastic cells are not known. This prompted us to study the plasma membrane transport of 5-ALA in a human adenocarcinoma cell line (WiDr). It is shown in this report that 5-ALA, but not 5-ALA esters are taken up by carrier systems transporting the β-amino acids β-alanine and taurine as well as γ-aminobutyric acid (GABA) in these cells.

EXPERIMENTAL

Cell Cultivation

Cells of an established line (WiDr), derived from a human primary adenocarcinoma of the rectosigmoid colon [5], were subcultured in RPMI 1640 medium (Gibco) containing 10 % fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. The cells were subcultured approximately twice a week (split ratio, 1:100) and maintained at 37°C and 5 % CO_2 in a humid environment.

Chemicals

δ-[4-^{14}C]-Aminolaevulinic acid (specific activity 47.6 mCi·mmol^{-1}) was purchased from NEN™ Life Science Products (Boston, USA). Unlabeled 5-ALA (Sigma, St. Louis, MO or Photocure, Norway) was dissolved in Dulbecco's PBS, RPMI 1640 medium without serum, or a buffer containing 10 mM HEPES, 150 mM NaCl, 1.2 mM $CaCl_2$, 0.64 mM $MgCl_2$, 6.0 mM KOH, and 5.0 mM D-glucose (named HEPES-buffer). pH was adjusted to 7.4 with 5 M NaOH. 5-[^{14}C]ALA was diluted 48-fold with this solution of cold 5-ALA. All other chemicals were of analytical grade and of highest purity commercially available. All amino acids used were L-stereoisomers.

Uptake Measurements

The cells (2.5-5 x 10^4 cells · cm^{-2}) were seeded into 24 well plates 2-3 days before experiments; nearly confluent layers of cells were incubated with radiolabelled material at the desired concentration under various conditions. The cells were either incubated in RPMI 1640 medium without serum, PBS, or in the HEPES-buffer. After treatment, the cells were kept on ice and washed four times in ice-cold PBS. The cells were dissolved in 200 µl 0.1 M NaOH. After 10 min of incubation 3 ml scintillation fluid (Opti-fluor, Packard) was added to the samples, and radioactivity was measured in a Packard Tri-Carb 4550 scintillation counter.

Measurements of Cellular PpIX Content

Cells (2.5-5 x 10^4cells · cm^{-2}) were seeded into 6 well plates 2-3 days before experiments and treated as described elsewhere. After treatment the cells were washed twice in ice-cold PBS and brought into a solution of 1.0 M $HClO_4$ in 50 % methanol by scraping with a Costar cell scraper. Cell debris was removed by centrifugation. PpIX was quantitatively extracted from the cells by this procedure [6]. The PpIX content of the samples was detected spectrofluorometrically using a Perkin Elmer LS50B spectrofluorometer. PpIX was excited at 408 nm and fluorescence measured at 605 nm. A long-pass cut-off filter (530 nm) was used on the emission side. A standard of known concentration was added to the samples at a concentration increasing the total fluorescence by approximately 50 %.

Protein determination

Protein was assayed by Bradford's method [7] using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany) with human serum albumin as standard.

Statistical analysis

Uptake measurements were routinely done in duplicate. Each experiment was repeated two or three times. The results are presented as means ± S.E.M. Linear regression analysis was performed by the method of least squares.

RESULTS

5-ALA uptake kinetics

5-ALA that has entered the cytoplasm may enter the heme synthesis pathway. In this study radioactively labelled 5-ALA has been utilised for measuring cellular uptake of 5-ALA. Some of the counts registered by the scintillation counter may thus stem from heme intermediates. However, this does not influence on the results since the uptake rates were independent of the presence of succinyl acetone, an inhibitor of the enzyme 5-ALA dehydratase, which converts 5-ALA into porphobilinogen [8].

Figure 1a shows the time-course of 5-ALA uptake at different concentrations of 5-ALA. At all concentrations (0.1-10 mM) uptake of 5-ALA was linear during the first 5 h. Cytotoxic effects were observed when the cells were treated for more than 3 hours with 20 mM of 5-ALA. The rate of 5-ALA uptake (from Figure 1a) were plotted as a function of 5-ALA concentration (Figure 1b). These results were fitted by regression analysis to the function y = ax/(b+x) according to the Michaelis-Menten function $v = V_{max}$ [S]/(K_m + [S]) in which v is the rate of 5-ALA uptake, V_{max} is the maximum rate of 5-ALA uptake, [S] is the concentration of 5-ALA, and K_m is the concentration of 5-ALA inducing ½ * V_{max}. The rate of 5-ALA uptake was well fitted to the above function (r^2 = 0.99) with K_m = 8.4 mM and V_{max} = 18 ± 0.9 nmol · (mg protein x h)$^{-1}$. Essentially identical results were obtained when calculating and plotting the data according to the Eadie-Hofstee equation (K_m = 10 ± 1.6 mM and V_{max} = 20.7 ± 2.5 nmol · (mg protein x h)$^{-1}$) (Figure 1c).

Temperature dependence of 5-ALA uptake

The influence of incubation temperature upon the uptake kinetics of 5-ALA was evaluated after 4 h of incubation with 1 mM 5-ALA in PBS containing 5 mM D-glucose (Figure 2a). The rate of 5-ALA uptake was found to be clearly temperature dependent, with negligible uptake at 0°C. The small fraction of 5-ALA associated with the cells at 0°C did not increase with time of incubation, and is therefore most likely due to 5-ALA associated with the plasma membrane. The activation energy for 5-ALA uptake was calculated by linear least-squares regression analysis of a plot of log k against 1/T according to the Arrhenius equation (Figure 2b):

$$\log k = \log A - \frac{E_a}{2.303RT} \quad :$$

in which k is the uptake of 5-ALA, A is the frequency factor and E_a is the activation energy. The Arrhenius plot revealed a change in slope at 32°C with activation energies of E_a = 112 kJ · mol^{-1} below 32°C and E_a = 26 kJ · mol^{-1} above 32°C (Figure 2b).

Na^+-dependence of 5-ALA uptake

The Na^+-dependence of 5-ALA uptake in WiDr cells was analysed by substituting NaCl with choline chloride (Figure 3). Incubation with 150 mM choline chloride for 3 h did not induce cytotoxic effects in the cells as revealed by microscopic inspection and propidium iodine staining. However, after 4 h of incubation a few cells started to

round up. Uptake of 5-ALA in WiDr cells was therefore analysed after 3 h of incubation. As seen in Figure 3, 5-ALA uptake in WiDr cells is highly Na^+-dependent and is reduced 9-fold in the absence of Na^+. Under these conditions about 2 nmol $(mg\ protein)^{-1}$ 5-ALA was associated with the cells after 3 h of incubation with 1 mM 5-ALA. This is a somewhat larger amount than what is expected to be surface-bound, indicating a minor Na^+-independent pathway. Measurements of PpIX formation in cells treated with 1 mM 5-ALA showed that PpIX formation was reduced by more than 60 % in the absence of Na^+ (data not shown).

The relationship between the uptake and the Na^+ concentration was found to be non-linear, indicating participation of more than one Na^+ per transport of one molecule of 5-ALA (Figure 3). To calculate the number of Na ions involved per transport cycle, the experimental data over a Na^+ concentration range of 60-150 mM were analysed by a Hill-type plot (v vs. $v/[S]^n$), as shown in Figure 3, *inset*. The plot gave a straight line with the best fit ($r^2 = 0.84$) when the Hill coefficient (n) was 3.4, suggesting that three Na ions are associated with the transport of one 5-ALA molecule.

Inhibition of 5-ALA uptake with α-amino acids

As seen by comparing results in Figure 1 with those in Figures 2 and 3, the rate of 5-ALA uptake seems to be about 2-fold higher in simple salt solutions than in the amino acid containing RPMI 1640 medium. In order to evaluate the possible uptake of 5-ALA through amino acid transporters, WiDr cells were incubated with 23 μM 5-[^{14}C]ALA and 10 mM of various amino acids (Figure 4). It was found that non-

labelled 5-ALA inhibited the uptake of 5-[^{14}C]ALA as expected for an active transport pathway. In contrast, 5-ALA methyl ester did not inhibit the uptake of 5-[^{14}C]ALA. The transport of 5-ALA was inhibited by basic amino acids and, with the exception of threonine, valine, and methionine, all the zwitterionic α-amino acids. The acidic amino acids aspartate and glutamate did not significantly attenuate the transport of 5-ALA.

Uptake through the β-amino acid and GABA transporter

5-ALA is a δ–amino acid with structural similarities to the β-amino acids β-alanine and taurine as well as GABA in that they all have amino and acid groups on opposite terminal parts of the molecules (Figure 5). It was found that all 3 amino acids inhibited 5-[^{14}C]ALA uptake more efficiently than 5-ALA itself, and equally or more efficiently than all the other amino acids investigated (Figure 6).

The β-amino acids and GABA transporters are characterised by their chloride-dependency [9,10]. Choride ions (Cl^-) were therefore substituted with a mixture of gluconate, NO_3^-, and SO_3^- salts [9], and the results clearly show that the 5-ALA uptake is Cl^--dependent (Figure 7). There appears to be a linear relationship between the [Cl^-] and the 5-ALA uptake ($r^2 = 0.994$), indicating a 1:1 Cl^-:5-ALA coupling ratio. Similar results were obtained when Cl^- was substituted with gluconate only (data not shown). It should however be noted that 5-ALA is delivered as 5-ALA·HCl, and when all Cl^- is removed from the medium there will still be about 1 mM Cl^- left in the medium.

The present results indicate that 5-ALA is to a large extent taken up by WiDr cells through the β-amino acid and GABA transporters. To investigate the importance of these transporters upon the rate of PpIX formation, cells were incubated with 0.3 mM 5-ALA in the presence or absence of 10 mM β-alanine (Figure 8). In these experiments β-alanine was found to reduce the 5-ALA-induced PpIX formation by 85%. In contrast, β-alanine had no significant effect on the PpIX formation in 5-ALA methyl ester or 5-ALA hexyl ester treated cells.

DISCUSSION

The data presented herein indicate that transport of 5-ALA into human adenocarcinoma cells (WiDr cells) is mediated by an active transport mechanism. The uptake was found to be saturable following Michaelis-Menten type kinetics (Figure 1), with an activation energy that is within the range of active transport (Figure 2). The transport was found to be Na^+- and Cl^--dependent and inhibited by several zwitterionic and basic amino acids, in particular the β-amino acids β-alanine and taurine as well as GABA (Figs.4,6,7). Furthermore, the transport has also recently been found to be energy-dependent [8]. The transporter(s) involved displays a low affinity (K_m = 8-10 mM), but at the same time a relatively high capacity (V_{max} = 18-20 nmol · (mg protein x h)$^{-1}$) for the uptake of 5-ALA in WiDr cells.

In WiDr cells PpIX formation increases with extracellular 5-ALA concentrations up to about 1 mM, above which PpIX formation is independent of the 5-ALA concentration [6]. It is here demonstrated that K_m for uptake of 5-ALA in WiDr cells, under similar conditions, is 8-10 mM. This means that at extracellular concentrations of 5-ALA above 1 mM, transport of 5-ALA over the plasma membrane is not the rate-limiting step in PpIX formation. Of the enzymes involved in the heme synthesis, porphobilinogen deaminase (PBGD) and coproporphyrinogen oxidase may be rate limiting steps in the formation of PpIX [11-13]. Thus, PBDG may constitute the rate-limiting step in the formation of PpIX at extracellular concentration of 5-ALA above 1 mM.

The calculated activation energy of 112 kJ · mol^{-1} for 5-ALA transport in the temperature range 15-32°C (Figure 2b) clearly exceeds that anticipated for simple diffusion, and is in the range for carrier-mediated processes (E_a > 30 kJ · mol^{-1} [14]). Above 32°C the uptake of 5-ALA was less temperature-dependent and the activation energy was about 26 kJ · mol^{-1}. This is an activation energy which is higher than that for simple diffusion (E_a <17 kJ · mol^{-1} [14]), but below the usual range for carrier-mediated processes. The observed discontinuity in the Arrhenius plot at 32°C may be interpreted as a phase transition in the membrane fluidity [15]. The phase transition temperature vary with the lipid composition of the membrane, e.g. for alanine transport it varied between 16°C in CHO cells enriched in oleate and 32°C in cells enriched in palmitate [16].

Separate transport systems usually serve for cationic, zwitterionic, and anionic amino acids in most mammalian cell types [17]. According to the classification by Stevens et al. [18], carrier systems for amino acids have been categorised into Na^+-dependent carriers (systems B, A, ASC, N, Gly, X_{AG}^-, β, IMINO and GABA) and Na^+-independent carriers (systems L, y^+, $b^{0,+}$, and x_c^-). The transport of 5-ALA was both Na^+- and Cl^--dependent (Figs. 3 and 6). Several of the Na^+-dependent amino acid transporters are most likely not specific transporters for 5-ALA: 1) 5-ALA was recently found not to influence on the uptake of methyl-AIB, specifically transported by the widespread system A [8]; 2) Threonine, but not glycine, reacts well with system ASC, while glycine, but not threonine, is a competitive inhibitor for 5-ALA transport [19](Figure 4). System ASC is relatively pH-insensitive and trans-stimulated, which is not the case for transport of 5-ALA [8]; 3) System Gly is specific

for glycine and sarcosine, while the transporter(s) for 5-ALA has a broad specificity [20]; 4) System X_{AG}^- has a specificity for glutamate and aspartate which do not significantly reduce the uptake of 5-ALA (Figure 4) [21]; 5) Systems N and N^m is specific for glutamine, histidine and asparagine, which also are inhibitors of 5-5-ALA uptake, but these transporters have only been found in liver and skeletal muscle [22]. 6) System IMINO does not seem to use neutral amino acids as substrates, which is in contrast to the 5-ALA transporter(s) [23]. However, both β–amino acid transporters, the GABA transporters, and system $B^{0,+}$, are dependent upon Na^+ and Cl^- [22,24-26], and are thus candidates for transport of 5-ALA: System $B^{0,+}$ has a broad specificity similar to that of the 5-ALA transporter, and it is also inhibited by β-alanine, but displays a lack of specificity for taurine at least in mouse blastocytes [27,28]. The observed inhibition of 5-ALA transport by β-alanine, taurine (substrates for system β) and GABA (Figs.6-8), indicate a transport of 5-ALA through system β and the GABA transporters, which have overlapping substrate specificities [29,30], i.e. β-alanine and taurine may be transported by the GABA transporters and GABA is a substrate for β-amino acid transporters. It was reported that transport of β-alanine, as found for 5-ALA in this study (Figure 4), was inhibited by lysine, leucine, glycine, serine, alanine and proline in the epithelium from rabbit distal ileum [31]. However, in the HT-29 human colon carcinoma cell line, transport of taurine was inhibited by β-alanine, but not by alanine and leucine [32]. Brush-border membrane vesicles from proximal intestine contain a β-alanine transporter with high specificity [9]. The uptake of β-alanine through this transporter was not inhibited by proline, lysine and aspartate. This is in contrast to the 5-ALA transport pointing towards tissue and species differences in transporter specificity. GABA transporters have been shown to be

expressed in many tissues outside CNS [10,33-38]. The GABA transporters GAT-2 and GAT-3 are inhibited 60 and 80% in the absence of Cl^- similar to the transport of 5-ALA (Figure 7), whilst that of GAT-1 is 95% inhibited [10]. Based on these considerations, it thus seems that the transport of 5-ALA may occur on both the β-amino acid transporter system β and GABA transporters, while transport on system $B^{0,+}$ can not be excluded. However, it should be emphasised that inhibition of β-alanine transporters could account for 85% of PpIX formed in cells treated with 0.3 mM 5-ALA (Figure 8), pointing towards the importance of β-alanine transporters for the uptake of 5-ALA.

Neoplastic cells exhibit increased demands for certain metabolites, including amino acids, which must be transported across the plasma membrane. This result in not only enhanced expression of amino acid transporters, but also in expression of isoforms not found in their normal counterparts [39,40]. In general, little is known about amino acid transport in human neoplastic cells and comparison with transporters in normal cells must therefore be executed with caution. Colon carcinoma cell lines, particularly HT-29 and Caco-2, have been shown to exhibit enterocyte-like differentiation features [41]. It has been documented that colon carcinoma cells (HT-29) express a common transporter for both taurine and β-alanine [32], indicating that a similar transporter may be involved in the transport of 5-ALA in the WiDr colon adenocarcinoma cells. There are so far no reports indicating GABA transporters in colon carcinomas, and the overlapping specificities of β-amino acid and GABA transporters suggest that GABA competes with 5-ALA for transport through a β-amino acid transporter in WiDr cells. However, the results from this study indicate that transport of 5-ALA by GABA

transporters in other cell types should be considered. Previous reports indicating that 5-ALA is transported by the GABA transporter UGA4 in *S.cerevisiae* support such a notion [42]. An enhanced expression of amino acid transporters may also account for accumulation of more PpIX in neoplastic tissues treated with 5-ALA than in their normal counterparts [2].

Systemic administration of 5-ALA to patients induces a significant decrease in systolic and diastolic blood pressure [43]. GABA regulates cardiovascular function via central and peripheral GABA receptors [44-46]. Intravenous administration of GABA lowers blood pressure and induces bradycardia [47]. Thus, the 5-ALA-induced lowering of blood pressure may be related to its binding to GABA receptors [48]. Furthermore, GABA transporters are found in peripheral neurons [49-51]. Thus, 5-ALA, but not 5-ALA esters (Figures 6 and 8), should be expected to be preferentially taken up into peripheral nerve endings. This may explain the clinical experience with the more severe pain reactions observed during light exposure of basal cell carcinoma lesions after topical application of 5-ALA as compared to 5-ALA methyl ester (A.Soler, J.Tausjø and T.Warloe, unpublished work). The lack of inhibitory effect of β-alanine upon 5-ALA ester-induced PpIX formation (Figure 8) and lack of inhibitory effect of 5-ALA ester on the transport of 5-ALA (Figure 4), indicate that 5-ALA esters are taken up by other transporters than those used for uptake of 5-ALA or that they may penetrate the plasma membrane by passive diffusion.

The present study document that amino acid transporters are involved in the uptake of 5-ALA in WiDr cells. Whether transporters of other metabolites like the apical

peptide transporters PEPT1 and PEPT2 as found in epithelial cells of the intestine and kidney [52,53] also are involved in transport of 5-ALA is not known. Furthermore, different transport mechanisms between 5-ALA and 5-ALA-esters may provide a molecular explanation for some of the differences observed with such compounds in clinical PDT.

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FIGURE LEGENDS

Figure 1. Time course and kinetics of 5-ALA transport in WiDr cells.

Uptake of 5-ALA was measured in RPMI 1640 medium over a concentration range of 0.1-10 mM as indicated in a) and analysed for cellbound 5-ALA as described in the Experimental section. The linear regression lines obtained in a) were used to plot the rate of 5-ALA uptake versus concentration of extracellular 5-ALA (b) and an Eadie-Hofstee plot (v/s versus v).

Figure 2. Effect of temperature upon 5-ALA transport.

WiDr cells were incubated for 4 h in PBS containing 1 mM 5-ALA and 5 mM glucose at the indicated temperatures and otherwise treated as described in the Experimental section (a). The results are also presented in an Arrhenius plot (b). Linear regression lines were obtained by the method of least squares (r^2 = 9.977 and r^2 = 0.941 in the regions 15-32°C and 32-42°C respectively). In the Arrhenius plot, binding of 5-ALA to the plasma membrane (at 0°C) was subtracted in order to assess only the uptake of 5-ALA.

Figure 3. Dependence of 5-ALA uptake upon Na^+-concentration.

WiDr cells were incubated with 1 mM 5-ALA in a HEPES-buffer in which the Na^+ concentration is varied by substituting NaCl with choline chloride and otherwise treated as described in the Experimental section. Uptake of 1 mM 5-ALA was measured after a 3 h incubation. Inset: Hill-type plot in which the

velocity (v, nmol · (mg protein x 3 h)$^{-1}$) was plotted against v · $[Na^+]^n$, with n=3.4, $r^2 = 0.84$).

Figure 4. Inhibition of 5-ALA uptake by various amino acids.

The average amount of 5-[^{14}C]ALA (23 μM) taken up by WiDr cells after 3 h in the presence of various amino acids (10 mM) is reported as the percentage of the mean uptake in the absence of any added non radioactive amino acid as indicated on the figure. The cells were incubated in a HEPES-buffer as described in the Experimental section.

Figure 5. Some compounds structurally related to 5-ALA and employed in the study.

Figure 6. Inhibition of 5-ALA uptake by β-alanine and taurine (a) and GABA (b). The average amount of [^{14}C]-5-ALA (23 μM) taken up by WiDr cells after 3 h in the presence of the indicated amino acids (10 mM) is reported as the percentage of the mean uptake in the absence of any added non radioactive amino acid as indicated on the figure. The cells were incubated in a HEPES-buffer as described in the Experimental section.

Figure 7. Dependence of 5-ALA uptake upon Cl^--concentration.

WiDr cells were incubated with 1 mM 5-ALA in a HEPES-buffer in which the Cl^- concentration is varied by substituting NaCl with Na gluconate, MgCl with $MgSO_4$ and CaCl with $Ca(NO_3)_2 \cdot 4H_2O$ and otherwise treated as described in

the Experimental section. Uptake of 1 mM 5-ALA was measured after a 3 h incubation.

Figure 8. Effect of β-alanine upon the accumulation of PpIX in WiDr cells treated with 5-ALA or 5-ALA esters.

The cells were treated with 0.3 mM 5-ALA, 0.3 mM 5-ALA methyl ester, or 4 μM 5-ALA hexyl ester for 4 h in RPMI 1640 medium without serum in the absence (black bars) or presence (grey bars) of 10 mM β-alanine.

Fig.1

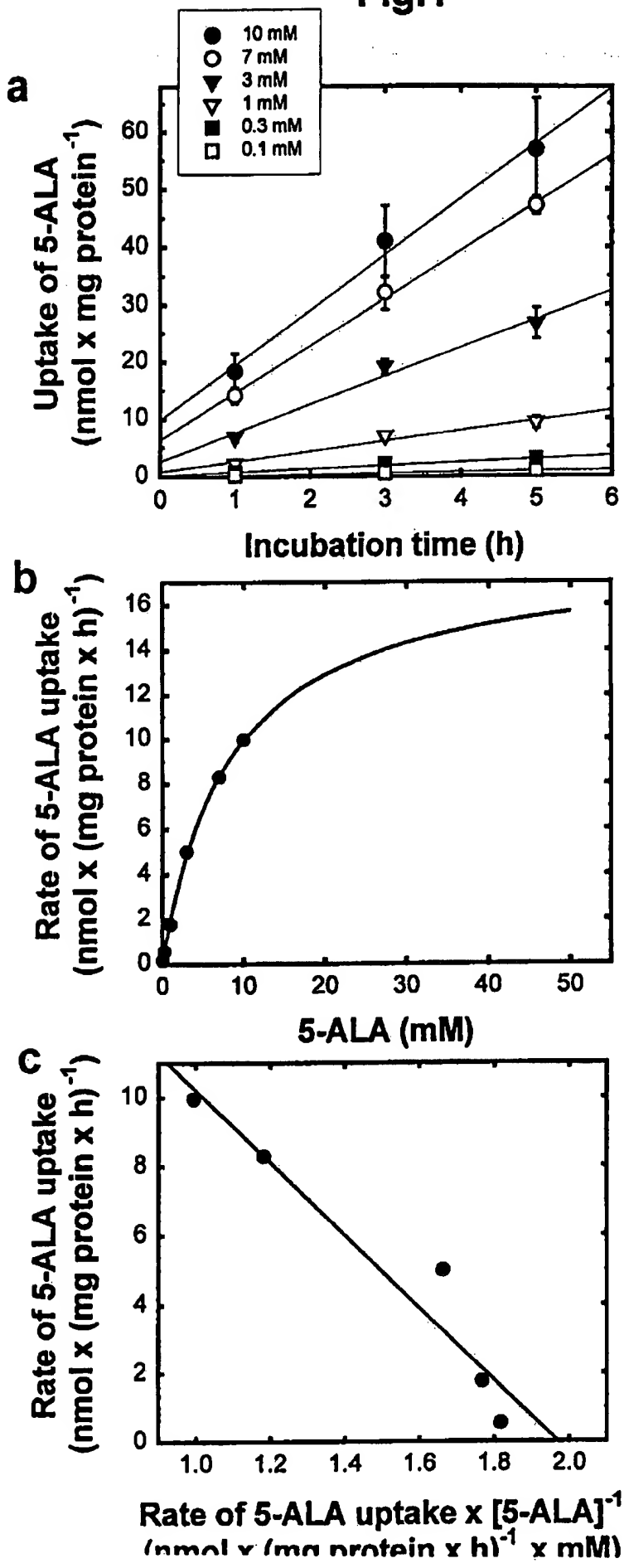

a
10 mM
7 mM
3 mM
1 mM
0.3 mM
0.1 mM
Uptake of 5-ALA (nmol x mg protein^{-1})
Incubation time (h)
b
Rate of 5-ALA uptake (nmol x (mg protein x h)$^{-1}$)
5-ALA (mM)
c
Rate of 5-ALA uptake (nmol x (mg protein x h)$^{-1}$)
Rate of 5-ALA uptake x [5-ALA]$^{-1}$
(nmol x (mg protein x h)$^{-1}$ x mM)

Fig.2

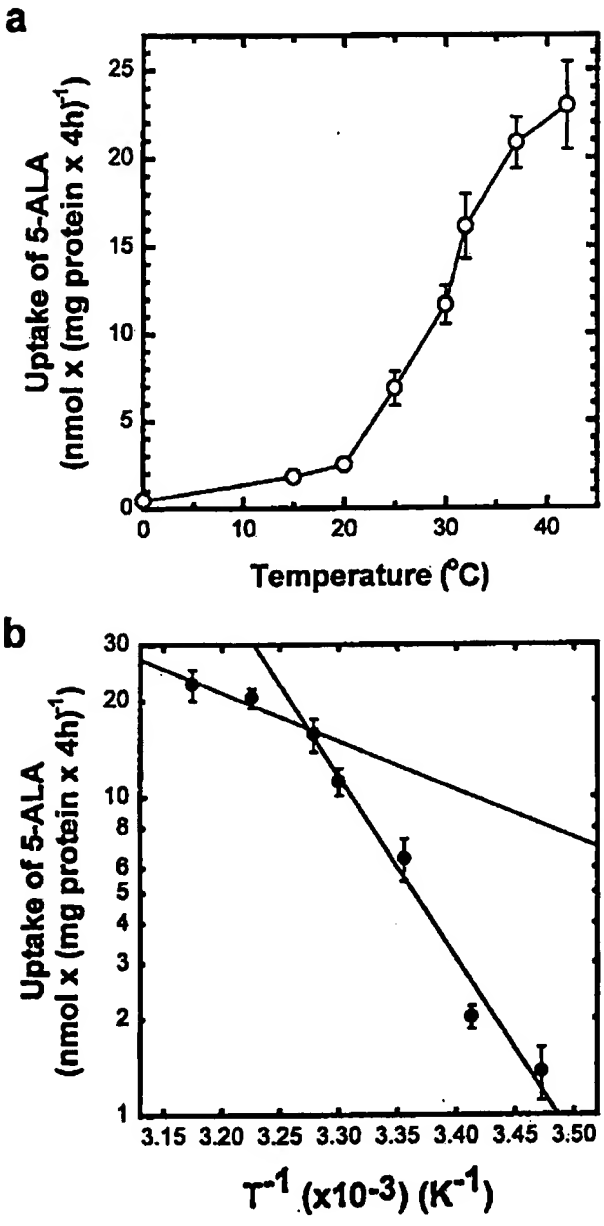

a
Uptake of 5-ALA
(nmol x (mg protein x 4h)$^{-1}$)
0
5
10
15
20
25
0
10
20
30
40
Temperature (°C)
b
Uptake of 5-ALA
(nmol x (mg protein x 4h)$^{-1}$)
1
2
3
4
5
6
8
10
20
30
3.15 3.20 3.25 3.30 3.35 3.40 3.45 3.50
T^{-1} (x10-3) (K^{-1})

Fig.3

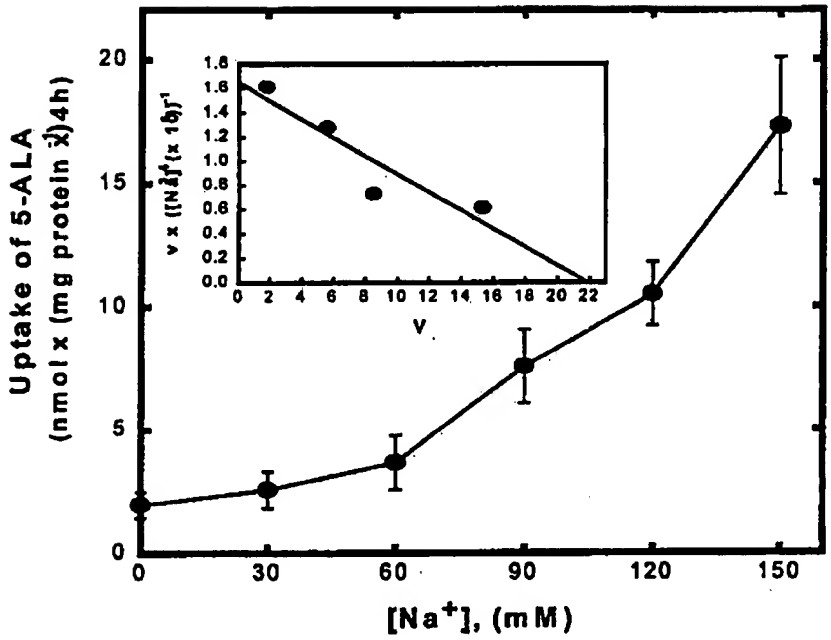

Uptake of 5-ALA
(nmol x (mg protein x 4h)
20
15
10
5
0
0
30
60
90
120
150
[Na+], (mM)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 2 4 6 8 10 12 14 16 18 20 22
V

Fig.4

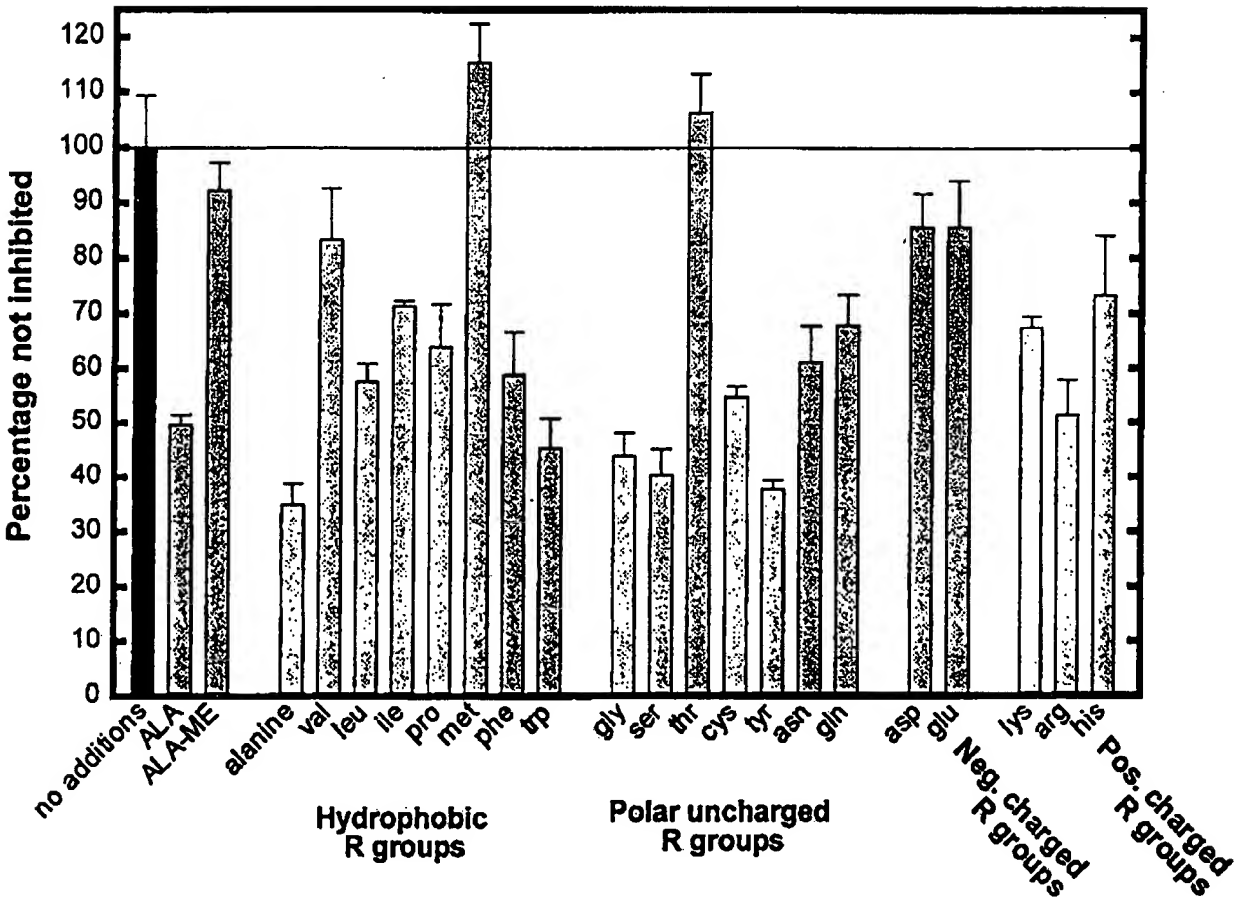

Percentage not inhibited
120
110
100
90
80
70
60
50
40
30
20
10
0
no additions
ALA
ALA-ME
alanine
val
leu
ile
pro
met
phe
trp
gly
ser
thr
cys
tyr
asn
gln
asp
glu
lys
arg
his
Hydrophobic R groups
Polar uncharged R groups
Neg. charged R groups
Pos. charged R groups

Fig.5

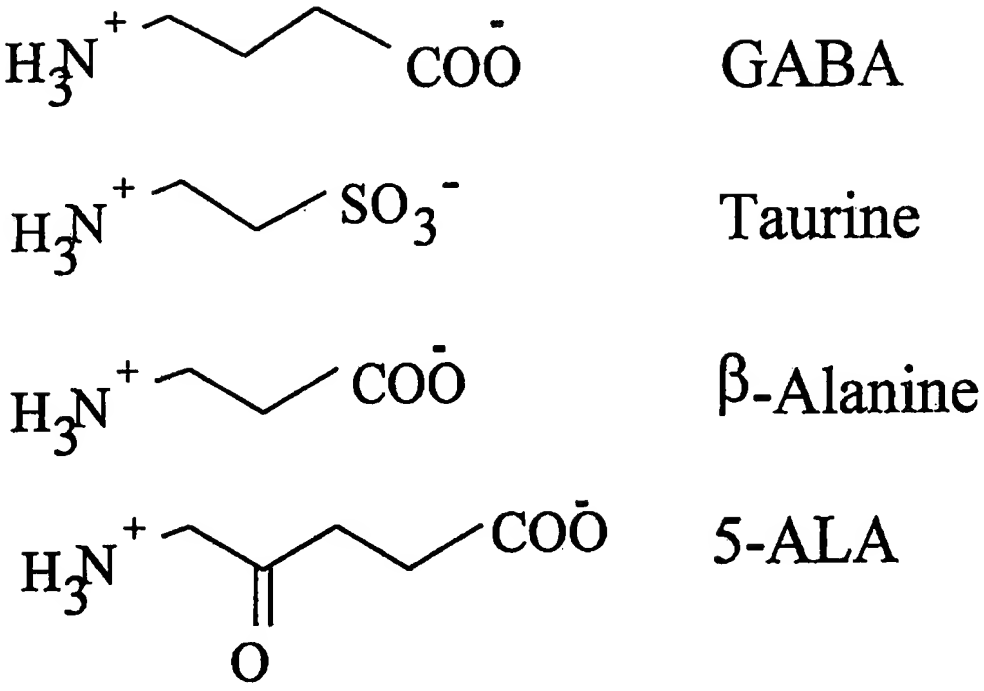


Fig.6

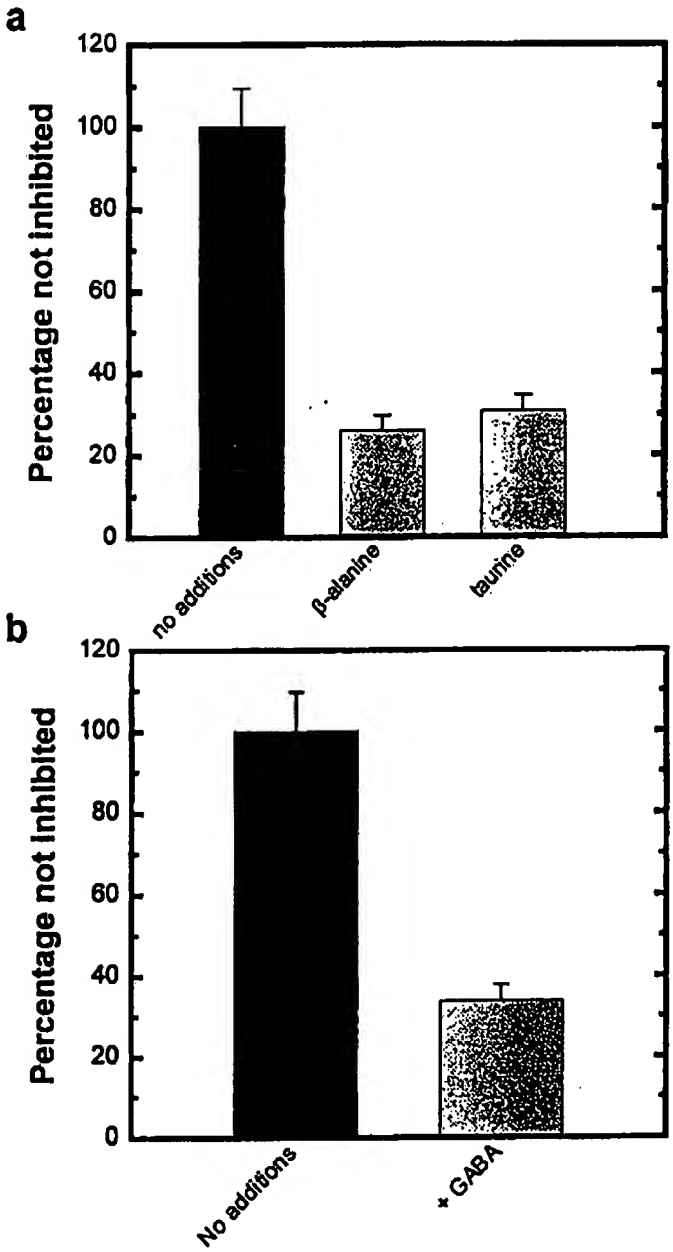

a
Percentage not inhibited
120
100
80
60
40
20
0
no additions
β-alanine
taurine
b
Percentage not inhibited
120
100
80
60
40
20
0
No additions
+ GABA

Fig.7

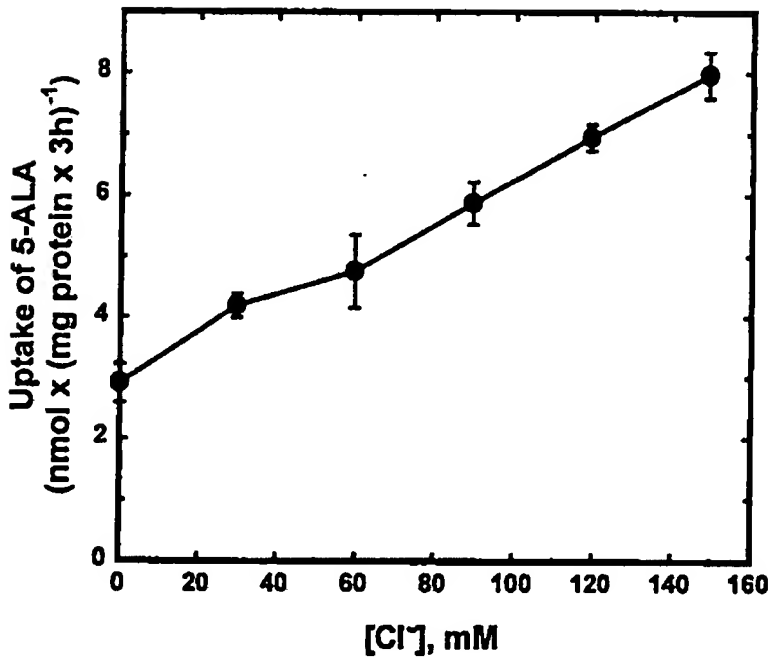
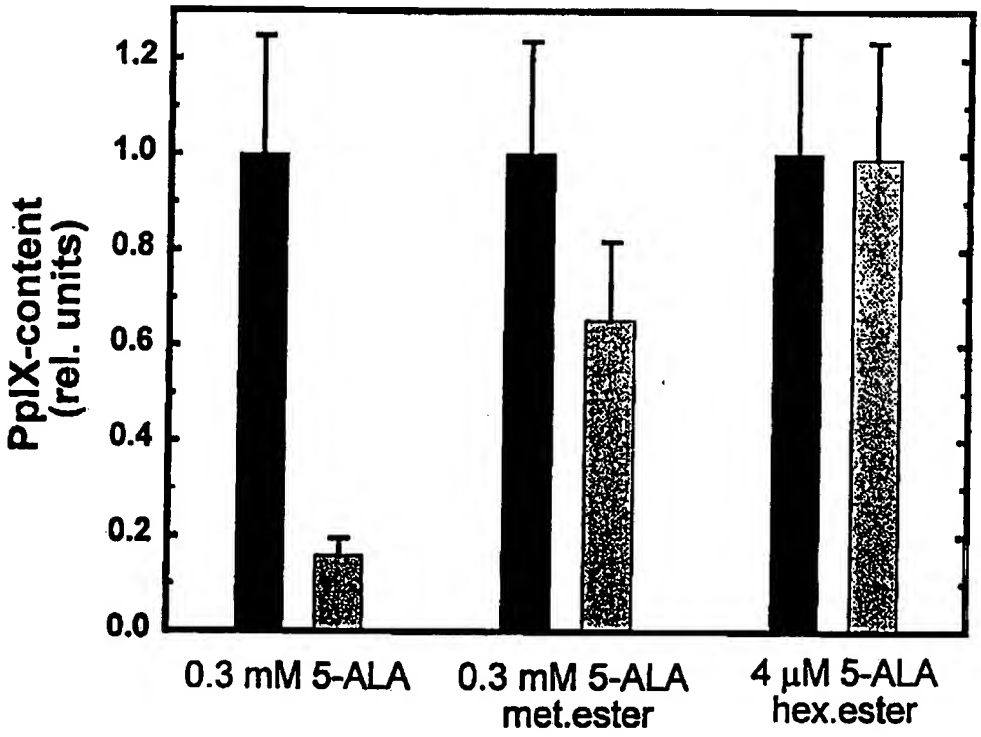

Uptake of 5-ALA
(nmol x (mg protein x 3h)$^{-1}$)
0
2
4
6
8
0
20
40
60
80
100
120
140
160
[Cl$^-$], mM

Fig.9 8


PpIX-content
(rel. units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.3 mM 5-ALA
0.3 mM 5-ALA
met.ester
4 µM 5-ALA
hex.ester

ANNEX 2

Manuscript of Rud & Berg entitled "Characterisation of the cellular uptake of 5-aminolevulinic acid"

CHARACTERISATION OF THE CELLULAR UPTAKE OF 5-AMINOLEVULINIC ACID.

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ABSTRACT

Photodynamic therapy with 5-aminolevulinic acid (5-ALA) is a promising alternative treatment for several types of cancer. This work represents a first approach to characterise the transport system for 5-ALA in human cancer cells, using WiDr cells from a primary adenocarcinoma of the rectosigmoid colon as a model system. The transport of 5-ALA in WiDr was found to be dependent on pH and temperature, and partially inhibited by inhibitors of the energetic metabolism. Although WiDr was shown to express System A, a common transport system for small aliphatic amino acids, the transport of 5-ALA in WiDr was not found to be mediated by this system.

Key Words: 5-aminolevulinic acid, photodynamic therapy, cellular uptake

1. INTRODUCTION

Photodynamic therapy (PDT) of malignant and nonmalignant lesions of skin and other organs is gradually becoming a more widespread treatment [1-3]. The treatment consists of the administration of a photosensitizer, usually followed by a periode of 4-72 hours, depending on the pharmacokinetics of the photosensitizer, to allow for optimal localization of the photosensitizer in tumor. Lesion(s) are then exposed to light corresponding to the appropriate wavelengths absorbed by the photosensitizer. The absorption of photons by the photosensitizer initiates a photochemical reaction, usually yielding singlet oxygen ($^1O_2$), which results in cytotoxity and regression of the lesion [4].

PDT with 5-aminolevulinic acid (5-ALA) is based on the application, either topically or orally of 5-ALA, an early precursor in the biosynthetic pathway for heme. Application of exogenous 5-ALA bypasses the natural regulation of the heme pathway and induces intracellular accumulation of fluorescent heme intermediates, mainly protoporphyrin IX (PpIX), in most tissues [1,5,6]. Due to the hydrophilic properties of 5-ALA, 5-ALA-PDT may be clinically limited by the rate of 5-ALA uptake in neoplastic cells and/or its penetration through tissue [7,8]. Whilst the ability of 5-ALA to penetrate skin has been somewhat studied [9,10], the transport mechanisms of 5-ALA are poorly understood.

So far, studies in yeast have established that 5-ALA uptake in *Saccharomyces cerevisiae* is mediated by the γ-aminobutyric acid-specific permease UGA4 [11-14]. In the

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bacteria *Salmonella typhimurium* [15] and *Echerichia coli* [16], 5-ALA is transported by a dipeptide permease. Aspects of 5-ALA uptake have also been studied in some animal and vegetable systems [17-22]. Although there recently has been two studies of 5-ALA uptake in a strain of mammalian epithelial cells in culture (CNM-I-221) [23,24] and also a study of 5-ALA uptake into two human and two rodent tumor cell lines in culture [25], little is known about the mechanisms of 5-ALA uptake in human and/or cancer cells. Thus, in the present study some aspects of the uptake mechanisms for 5-ALA in a human adenocarcinoma cell line have been characterized.

2. MATERIALS AND METHODS

Cell Cultivation. Cells of the established line WiDr, derived from a human primary adenocarcinoma of the rectosigmoid colon [26], were subcultured in RPMI 1640 medium (Gibco) containing 10 % fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. The cells were subcultured approximately twice a week (split ratio, 1:100) and maintained at 37°C and 5 % CO_2 in a humid environment.

Chemicals. δ-[1-^{14}C]-Aminolevulinic acid (specific activity 47.6 mCi/mmol) and α-[1-^{14}C]-methylaminoisobutyric acid (specific activity 56.0 mCi/mmol) were purchased from NEN™ Life Science Products (Boston, USA). Unlabeled 5-ALA was dissolved in PBS or medium without serum, and pH was adjusted to 7.4 with 5 M NaOH. 5-[^{14}C]ALA was diluted with this solution of cold 5-ALA to provide a 1.0 mM stock solution with a specific activity of 1 µCi/ml. All other chemicals were of analytical grade and commercially available.

Incubation media. In most of the experiments, with exceptions listed below, RPMI 1640 medium without serum was used as incubation medium. The pH dependent uptake of 5-ALA was investigated in a buffer containing 10 mM HEPES, 150 mM NaCl, 1.2 mM $CaCl_2$, 0.64 mM $MgCl_2$, 6.0 mM KOH, and 5.0 mM glucose; pH was adjusted with HCl and NaOH. The uptake of (N-methylamino)-α-isobutyric acid (methyl-AIB) as well as the experiments with efflux and exchange of preloaded 5-ALA were assayed in PBS supplemented with 5 mM glucose.

Uptake Measurements. 2.5-5 x 10^4 cells were seeded into 1 cm^2 dishes on 24 well plates 2-3 days before experiments; nearly confluent layers of cells were incubated with radiolabeled material at the desired concentration under various conditions in the absence of serum. After treatment, the cells were kept on ice and washed four times in ice-cold PBS. The cells were dissolved in 200 µl 0.1 M NaOH. After 10 min of incubation 3 ml scintillation fluid (Opti-fluor, Packard) was added to the samples, and radioactivity was measured in a Packard Tri-Carb 4550 scintillation counter. Counts were converted to moles, and the results were corrected for protein contents.

Efflux and exhange of preloaded 5-ALA. Succinyl acetone (SA), an inhibitor of 5-ALA dehydratase [27] was employed when studying efflux and exchange of 5-ALA with 5-ALA in the extracellular medium. The addition of SA to the medium inhibits PpIX formation almost entirely. WiDr-cells were seeded out as described above, and

preincubated with 2 mM SA for 30 minutes before experiments. Content of radioactivly labeled material was determined in both medium and cells.

Measurements of Cellular PpIX Content. 2.5-5 x 10^5 cells were seeded into 10 cm^2 dishes on 6 well plates 2-3 days before experiments; nearly confluent layers of cells were incubated with 5-ALA under various conditions in the absence of serum. After treatment the cells were washed twice in ice-cold PBS and brought into a solution of 1.0 M $HClO_4$ in 50 % methanol by scraping with a Costar cell scraper. Cell debris was removed by centrifugation. PpIX was quantitatively extracted from the cells by this procedure. The PpIX content of the samples was detected spectrofluorometrically using a Perkin Elmer LS50B spectrofluorometer. The PpIX was excited at 408 nm and fluorescence measured at 605 nm. A long-pass cut-off filter (530 nm) was used on the emission side. A standard of known concentration was added to the samples at a concentration increasing the total fluorescence by approximately 50 %. Results were corrected for protein contents.

Protein determination. Protein was assayed by Bradford's method [28] using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany) with human serum albumin as standard.

Statistical analysis. Uptake measurements were routinely done in duplicate. Each experiment was repeated two or three times. The results are presented as means ± S.E.M.. Linear regression analysis was performed by the method of least squares.

3. RESULTS

Time-course of 5-ALA uptake. The time dependent accumulation of 5-ALA is shown in Fig. 1. The cells were incubated with 1.0 mM 5-ALA for up to 8 hours. At time zero the cells were rapidly flushed with medium containing radiolabeled 5-ALA, immediately placed on ice, and washed with ice-cold PBS as described in Materials and Methods.

The WiDr cells accumulated 5-ALA at a linear rate. The rate of 5-ALA uptake was 2.3 nmol/hour/mg protein by linear regression analysis.

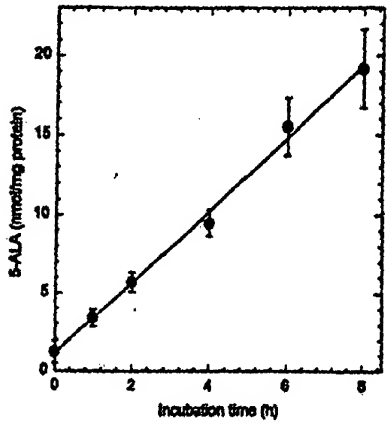

Fig. 1. Time course of 5-ALA uptake in WiDr cells. The cells were incubated with 1 mM 5-ALA for the indicated period of time as described in Materials and Methods.

Efflux and exhange of preloaded 5-ALA. In order to inhibit metabolization of 5-ALA, the cells were treated with 2 mM succinyl acetone (SA) which almost completely inhibited PpIX formation upon exposure to 1 mM 5-ALA (data not shown). When SA-treated cells were incubated with 10 mM 5-ALA for 4 hours, and then further incubated in the absence of 5-ALA for 4 hours, the PpIX formation was about 13 % of that in cells treated with 10 mM 5-ALA alone for 8 hours. Uptake of 1 mM 5-ALA over a 4 hour period was not affected by the precence of 2 mM SA (data not shown).

As shown in Fig. 2a there is a notable efflux of [^{14}C]5-ALA from SA-treated cells preloaded with 1 mM 5-ALA for 4 hours. The rate of efflux of 5-ALA seemed to be independent of extracellular 5-ALA concentrations up to 10 mM. Additionally, the uptake of 1.0 mM 5-ALA (Fig. 2b) was not influenced by preloading SA-treated cells with 10 mM unlabeled 5-ALA for 4 hours, indicating that the uptake of 5-ALA is not influenced by intracellular 5-ALA.

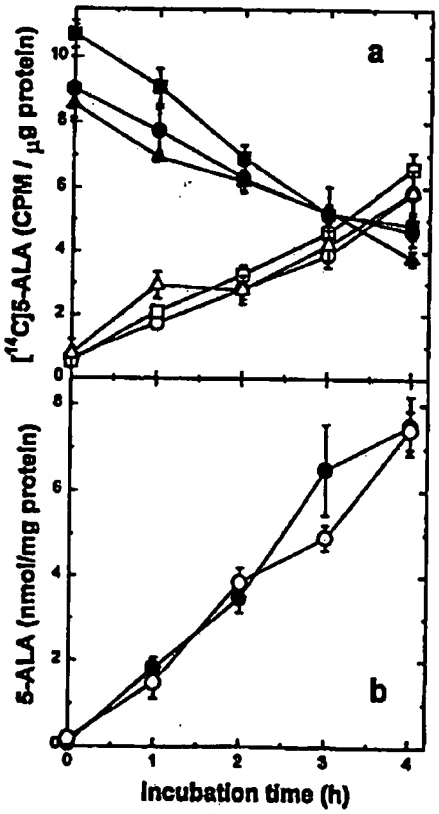

Fig. 2. Efflux and exchange of 5-ALA in WiDr cells. In (a) the cells were preloaded with 1 mM 5-ALA, including [^{14}C]5-ALA, and cellular efflux measured in the absence (●, ○) or presence of 1 mM (■, □) or 10 mM (▲, △) unlabeled 5-ALA in the medium by measuring radioactivity both in the cells (closed symbols) and in the medium (open symbols). In (b) uptake of 1 mM 5-ALA was compared in untreated cells (●) and cells preincubated with 10 mM unlabeled 5-ALA for 4 hours (○). The cells were initially treated with 2 mM SA as described in Materials and Methods.

absence ○
1mM □
10mM △

Temperature dependency of 5-ALA uptake. The time dependent uptake of 5-ALA at 4°C and 37°C were compared as shown in Fig. 3. Uptake of 5-ALA in WiDr cells were substantially attenuated by lowering the incubation temperature to 4°C. Linear regression analysis indicate no increase in 5-ALA uptake at 4°C with increasing incubation time, thus suggesting that binding of 5-ALA to the plasma membrane may be the cause of the cell associated 5-ALA (0.26 nmol/mg protein) after incubation at 4°C.

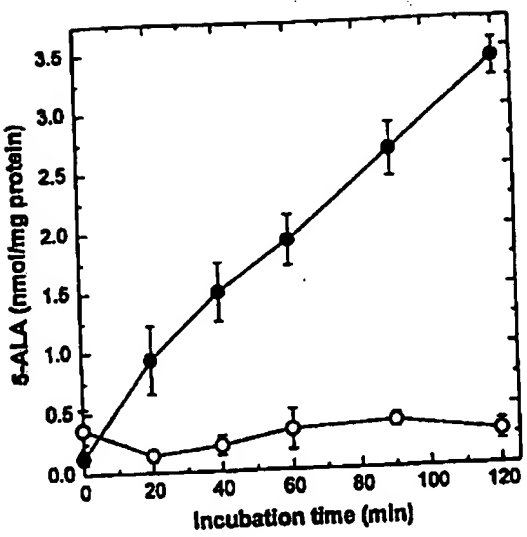

Fig. 3. Effect of temperature on 5-ALA uptake. WiDr cells were incubated with 1.0 mM 5-ALA at 4°C (○) and 37°C (●) for the indicated period of time as described in Materials and Methods.

Effect of external pH. The uptake of 5-ALA was highly pH dependent with an optimum around pH 7.5 (Fig. 4). From pH 6.5 to pH 7.5 the accumulation of 5-ALA increased four times. The accumulation of 5-ALA did not increase from pH 6.0 to pH 6.5.

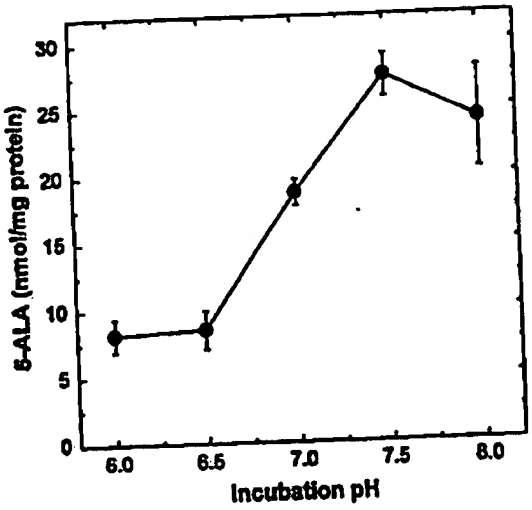

Fig. 4. Effect of external pH an 5-ALA uptake. WiDr cells were incubated with 1.0 mM 5-ALA and uptake was measured after 4 hours of incubation as described in Materials and Methods.

Inhibitors of energy metabolism. To assess the energy dependence of 5-ALA uptake 5-ALA entrance was measured in the presence of metabolic inhibitors. Treatment of the cells with sodium azide (10 mM) and 2-deoxy-D-deglucose (100 mM) for 1 hour reduced the ATP level in the cells to less than 3 %. Upon further incubation in the presence of both 1 mM 5-ALA and metabolic inhibitors for 2 hours the 5-ALA uptake was reduced by 44% as compared with cells treated with 5-ALA only. The plasma membrane was still intact after treatment with metabolic inhibitors as revealed by the propidium iodine exclusion method [29] (data not shown).

System A. As described above, the transport of 5-ALA in WiDr cells occurs in both directions, it is dependent on pH and temperature, and it is most likely energy dependent. These findings are consistent with the amino acid transporter system A [30], which transports small aliphatic amino acids and occurs in nearly all cell types. The non-metabolizable alanine analogue (N-methylamino)-α-isobutyric acid (methyl-AIB) is a substrate for this system. Cellular uptake of 0.05 mM [^{14}C]methyl-AIB revealed the existence of System A for amino acid transport in WiDr (Fig. 5). The uptake of [^{14}C]methyl-AIB was inhibited by the addition of a high concentration (20 mM) of unlabeled methyl-AIB, but not by an equimolar amount of 5-ALA, thus indicating that 5-ALA is not taken up by System A in WiDr cells.

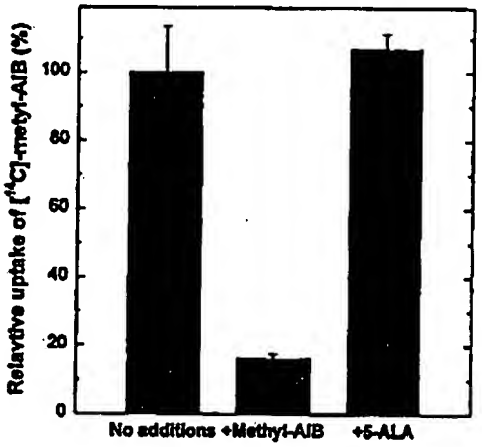

Fig. 5. Effect of 5-ALA and methyl-AIB on uptake of [^{14}C]methyl-AIB uptake. The cells were incubated for 3 minutes with 0.05 mM [^{14}C]methyl-AIB alone or in the precence of 20 mM unlabeled 5-ALA or methyl-AIB as indicated on the figure.

4. DISCUSSION

The initial step in the 5-ALA-induced synthesis of porphyrins is the penetration of 5-ALA through the plasma membrane. Since 5-ALA is a hydrophilic molecule and hydrophilic compounds poorly cross biological barriers with lipophilic properties, such as cellular membranes, the clinical usefulness of 5-ALA may be limited by the low rate of cellular uptake. This work represents a first approach to characterise the transport system for 5-ALA in human cancer cells, using WiDr cells as a model system.

Our results indicate a bi-directional, temperature- and pH-dependent uptake mechanism, which is at least partially energy dependent. The results show that the mechanisms for uptake of 5-ALA in WiDr cells exhibit many of the properties characteristic of an active transport system [31]. The rate of 5-ALA uptake and the amounts of 5-ALA accumulated in the WiDr cells are in accordance with the results of others [18,23].

After 5-ALA is taken up by the cells, it enters the heme biosynthesis pathway, and is converted into porphyrins and heme. Previous experiments performed on WiDr cells have shown that PpIX is the only photosensitizer which is formed in measureable amounts after 5-ALA treatment [32], and that there is formed about 40 % heme relative to PpIX after 4 hours of incubation of WiDr cells with 1.0 mM 5-

ALA[33]. Thus, after 4 hours of incubation with 1 mM 5-ALA 0.46 nmol/mg protein porphyrin molecules have been formed. This indicates that about 40% of the 5-ALA taken up by the cells is converted into PpIX.

In order to avoid ^{14}C counts from PpIX and other metabolites that may enter the medium when studying efflux of [^{14}C]5-ALA, succinyl acetone, an inhibitor of heme biosynthesis was employed. This proved relatively effective, since PpIX formation was reduced by 87 %. The transport of 5-ALA in WiDr cells proved to be bi-directional, proceeding equally well in both directions, both into and out of the cells. Also, the efflux of 5-ALA seemed unaffected by the extracellular 5-ALA concentration, as did the uptake of 5-ALA seem unaffected by the intracellular 5-ALA concentration used in these experiments. These results are contrary to the findings of Bermudez Moretti *et al.* [11] who found no efflux from preloaded cells and no exchange with external 5-ALA in *S. cerevisiae*.

The uptake of 5-ALA was highly temperature dependent, incubation of the cells at 4°C inhibited the uptake of 5-ALA almost completely compared to the uptake at 37°C. Inhibition of cellular uptake at low temperatures usually rules out passive diffusion as means of uptake [34], suggesting instead an active transport mechanism for the uptake of 5-ALA. These results differ from those of Gibson *et al.* [25]. They found no difference in uptake of radiolabeled 5-ALA between human or rodent tumor cells incubated either at 4 or 21°C *versus* cells incubated at 37°C [25], suggesting that an energy dependent carrier was not involved.

The rate of 5-ALA uptake was found to be pH dependent (Fig.4) . The optimal extracellular pH-value for 5-ALA uptake was around pH 7.5. The rate of uptake decreases above and below pH 7.5. The shape of the curve in Fig. 4 is almost identical to the shape of the curve for pH-dependent PpIX formation in WiDr cells found by Bech *et al.* [35], with the exception of an observed further decrease in PpIX formation below pH 6.5. 5-ALA is a water-soluble zwitterion [36], with pK_a values of 4 for the carboxylic acid groups and 8.9 for the amino group. Thus, it is not likely that the pH-dependency of 5-ALA uptake is due to changes in the amounts of different ionic species of 5-ALA [35]. Instead the pH-dependent variations in the uptake of 5-ALA might instead be due to a pH dependent transporter.

Incubation of WiDr with 5-ALA in the presence of metabolic inhibitors yielded a 44 % decrease in the uptake of 1 mM 5-ALA. This inhibition of the 5-ALA transport indicates that the process is (at least partially) energy dependent. Energy dependence of 5-ALA uptake has also been demonstrated earlier [17,21-23].

Even though the WiDr cells were shown to express system A [30], the uptake of 5-ALA was clearly not mediated by this transport system (Fig.5).

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